Title:

Persistent organic pollutant burden, experimental POP exposure and tissue properties affect metabolic profiles of blubber from grey seal pups.

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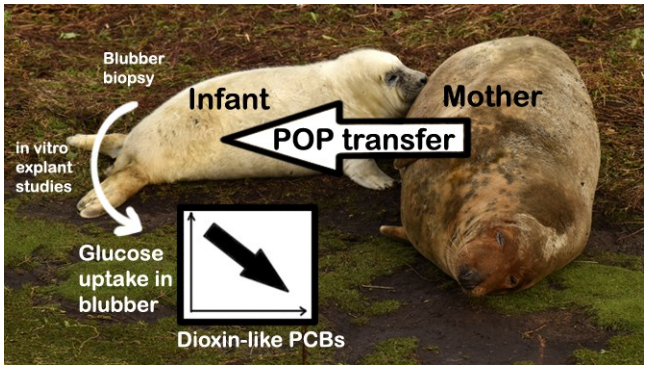
Keywords:

Dioxin-like PCBs; glucose uptake; lipolysis; lactate production; blubber depth; energetic state; environmental contamination; fasting; feeding

Abstract

Persistent organic pollutants (POPs) are toxic, ubiquitous, resist breakdown, bioaccumulate in living tissue and biomagnify in food webs. POPs can also alter energy balance in humans and wildlife. Marine mammals experience high POP concentrations, but consequences for their tissue metabolic characteristics are unknown. We used blubber explants from wild, grey seal (*Halichoerus grypus*) pups to examine impacts of intrinsic tissue POP burden and acute experimental POP exposure on adipose metabolic characteristics. Glucose use, lactate production and lipolytic rate differed between matched inner and outer blubber explants from the same individuals and between feeding and natural fasting. Glucose use decreased with blubber dioxin-like PCBs (DL-PCB) and increased with acute experimental POP exposure. Lactate production increased with DL-PCBs during feeding, but decreased with DL-PCBs during fasting. Lipolytic rate increased with blubber dichlorodiphenyltrichloroethane (DDT) and its metabolites (DDX) in fasting animals, but declined with DDX when animals were feeding. Our data show that POP burdens are high enough in seal pups to alter adipose function early in life, when fat deposition and mobilisation are vital. Such POP-induced alterations to adipose glucose use may significantly alter energy balance regulation in marine top predators with the potential for long term impacts on fitness and survival.

TOC/Abstract Art



Introduction

The impact of environmental and organismal accumulation of man-made chemicals is a growing global challenge[1,2]. Persistent organic pollutants (POPs) are toxic[3], accumulate in living tissues[4], and resist biological and chemical degradation[5]. Widespread bans on their production initially lowered environmental POP levels[6-9], but reductions have slowed or ceased more recently[10-13]. Legacy POPs are thus ubiquitous in the marine environment and remain a serious concern for top predators, in which biomagnification produces the highest burdens[4,11,14-16].

At high exposure levels POPs, such as polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs) and organochlorine pesticides (OCPs), including dichlorodiphenyltrichoroethane (DDT), impair fertility, reproductive function, skeletal and neurological development, immunity and thyroid function in humans and wildlife[17-24]. Recently, several POPs have also been implicated as lipid disruptors in humans and rodents[25,26]. In fish and zooplankton, POPs can increase fat deposition and lipogenic gene expression[27-31]. Conversely, exposure to POPs such as dioxins, lindane and OCPs can cause rapid energy reserve depletion, termed 'wasting syndrome'[32-35]. These apparently conflicting effects of different POPs on a variety of energetic processes highlight the need for further investigation of pollutant mixtures, doses and exposure routes, and their physiological consequences for organisms in natural environments.

POP-induced alterations to energy balance may have significant individual and population level consequences because fatness is often positively associated with reproductive fitness and survival[36]. This is particularly true in marine mammals[37-39], which rely heavily on subcutaneous fat reserves for insulation[40-41] and as a metabolic fuel when food is scarce. Many marine mammal species also undergo natural periods of

fasting during moulting, breeding, migration, or the postweaning fast that occurs in many phocid pups[37,42-46].

Marine mammals typically experience exceptionally high POP burdens due to their high fat content and top trophic level, and are regarded as sentinels of marine pollution[14,16]. POPs are transferred to suckling young as they undergo rapid fat accretion[47]. In fasting individuals, less hydrophobic POPs are released into the blood stream as lipid is mobilised and more recalcitrant POPs concentrate in the remaining blubber[47-54]. Phocid pups thus experience high POP exposure during both feeding and fasting, two fundamentally important periods for adipogenesis, fat mobilisation and maturation of energy balance pathways. Their reliance on adipose tissue to fuel metabolism during fasting or while searching for food in changing environments[55] place these top predators at high risk from adverse effects of POP exposure as a result of impaired fat cell function.

Marine mammals show structural changes in fatty acid (FA) content and thermal properties with blubber depth[54,56-61]. Effects of blubber depth and nutritional state on metabolic function and sensitivity to POP exposure have not been explored. High POP levels are associated with altered adipose gene expression in seals[62-64], polar bears (*Ursus maritimus*)[55], and killer whales (*Orca orcinus*)[65]. POP cocktails from polar bear fat can induce adipogenesis in mouse (*Mus musculus*) pre-adipocyte cell lines and primary polar bear pre-adipocytes[66]. However, the consequences of POP-induced gene expression changes for fat tissue function are unknown.

The major functions of fat tissue are to store lipid and release it for use as metabolic fuel. The balance between lipogenesis and lipolysis depends on the nutritional state of the organism and the hormonal milieu to which the tissue is exposed, and may be influenced by POPs. Glucose uptake, lactate production and glycerol release

97 are important metabolic properties of adipose tissue that contribute to whole animal
98 glucose homeostasis and energy balance. Adipose tissue synthesises triacylglycerol
99 (TAG) from glucose[67,68], such that glucose uptake rates can be used to investigate
100 metabolic function of adipose tissue. Glucose uptake is altered by POPs at tissue and the
101 whole animal level in other animals[69-87]. Of glucose taken up by adipose, 20% is stored
102 as TAG, and 70% is used to synthesise lactate[67]. Lactate production is thus an important
103 route of glucose disposal and may be influenced by POP exposure. Glycerol is produced
104 during lipolysis[88], and as a by-product of glycolysis[68], and is thus a major secretory
105 product of adipose tissue. Unlike FA, glycerol cannot be re-esterified by adipose *in*
106 *vitro*[89,90], making its production a useful marker of lipolytic[88], and, to some extent,
107 glycolytic[68] activity.
108 We measured glucose uptake, lactate production and glycerol release as indices
109 of metabolic activity in blubber by employing an explant approach we have developed
110 using blubber biopsies from wild grey seals (*Halichoerus grypus*)[91]. Our approach
111 allows experimental work to be performed *in vitro* in remote field locations to
112 investigate fat tissue function[91], which, in marine mammals, was previously limited to
113 measurement of enzyme activities[92]. Here, we used blubber explants from wild grey seal
114 pups, which remain on land during both feeding (during suckling) and fasting (during
115 the post-weaning fast). We investigated whether metabolic properties of blubber differ
116 by tissue depth, nutritional state and intrinsic POP burden. We also investigated acute
117 changes in blubber metabolic characteristics in response to exogenous experimental
118 treatments with high, but biologically relevant, POP concentrations.
119
120
121

Methods

Study site and animals

Field work was conducted on the Isle of May, Scotland (56◦11'N, 02◦33'W) from 7th November to 18th December 2016 under permit from Scottish Natural Heritage (SNH). Grey seal females that were part of a long-term study were observed from when they came ashore, and the date of birth of their pups was recorded. Pups were included if they were at an appropriate predicted mass for their age (number of days post-partum) late in the suckling period and if they did not have any obvious indications of ill health. We collected blubber biopsies for POP analysis and explant culture from 27 pups late in the suckling period(day 15). Pups were then left to wean naturally from their mothers, which occurred at approximately 18 days old. We then attempted to resample the same pups once during the postweaning fast (day 12 after weaning). As pups were still feeding for several days between the two sampling events, additional mass gain and increases in blubber lipid percentage were possible between the feeding/fasting sampling points. Four pups were removed from the study because they developed an unrelated infection. This gave a total of 27 pups (9 female and 18 male) sampled while feeding and 23 (7 female and 16 male) of the same animals sampled again during fasting.

Pups were defined as weaned based on daily observation to determine maternal departure date[43]. Pups were then transferred to a large (~600m^2) fenced off area of the island, away from the main colony, with access to a pool of fresh water, in accordance with UK Home Office guidelines and based on prior studies[43,93].

The sex of all pups was determined at early lactation (~ day 5 of suckling). Pups were weighed at early and late lactation; when they were initially transferred into the

147 pen (within 2 days after weaning); and every 5 days in the pen. Pups less than 30 kg at
148 weaning were excluded from the study. To ensure pups were of a viable departure mass,
149 early release criteria were set at loss of 25% of weaning mass or reaching 30kg,
150 whichever was the higher mass[43]. It was not necessary to release any of the study
151 animals early. All pups were released into the colony with ready access to the sea.
152 All animal procedures were performed under the UK Home Office project
153 licence #70/7806 and conformed to the UK Animals (Scientific Procedures) Act, 1986.
154 All research received prior ethical approval from Abertay University and the University
155 of St Andrews Animal Welfare and Ethics Committee.
156
157 *Biopsy sampling*
158 Biopsy sampling, transport, explant creation and culture protocols were adapted
159 from a previous study[91]. Prior to sampling, pups were given a mass-specific intravenous
160 dose of Zoletil™ and subcutaneous injections of Lignol™ at the biopsy sites. Three full
161 depth blubber biopsies, one 6 mm and two 10 mm, were taken[94]. The 6 mm biopsy was
162 immediately wrapped in foil and frozen at -20 ºC for POP analysis[47,50]. The two 10 mm
163 biopsies were cut immediately, using sterile surgical scissors, into 'inner' (blubber
164 closest to muscle layer) and 'outer' (blubber closest to the skin) tissue, and placed into
165 separate 15 ml centrifuge tubes filled with warm (37 ºC) sterile Krebs Ringer solution
166 (pH 7.4; NaCl; 7.89 g/l; KCl; 0.373 g/l; $MgSO_4$;0.12 g/l; K_2HPO_4; 0.07 g/l; Glucose;
167 0.99 g/l; HEPES; 4.77 g/l; CaCl; 0.11 g/l[95] with 1% Antibiotic Antimycotic Solution
168 (all chemicals supplied by Sigma- Aldrich) for transport back to the field laboratory. A
169 single hidden suture (Ethicon Vicryl Suture (W9114) Violet, 3-0, 20mm, 75cm with
170 VICRYL Suture 1/2 circle 'Taper Point Plus' Needle) was used to close 10 mm biopsy
171 sites , either vertically or horizontally through the skin[96]. Pups were observed remotely

on a daily basis, and biopsy sites checked at each reweighing to document healing. The second sample was not taken until all the previous biopsy sites had healed.

Explant culture and POP exposure

All biopsies were returned to the field laboratory, processed and placed in the incubator within 30 minutes of collection, which is within timeframes used in prior explant studies on livestock[97,98] and wild seals[91]. Upon arrival, tissue was washed with 1 ml sterile, warm Krebs Ringer buffer. Visible hair, muscle or blood contaminated tissue was removed. Tissue was minced using a sterile scalpel into 5-10 mg pieces, and 100 mg portions of tissue were weighed out in a sterile Petri dish. The 100 mg explants were dispensed into pre-prepared 12 well plates warmed to 37ºC in a sterile, non-ducted PCR hood . In total, 1500µl of either complete cell culture media (medium 199, Hanks' Balanced Salts with 1% Antibiotic Antimycotic Solution, 1% FA supplement and 5% charcoal stripped foetal bovine serum) or complete media containing 150ng/ml POP mix (PCB Aroclor standard mix 1 (Sigma-Aldrich), containing Aroclor 1016; 500 µg/mL, Aroclor 1260; 500 µg/mL, Decachlorobiphenyl (PCB 209); 50 µg/mL and 2,4,5,6-Tetrachloro-m-xylene (an OCP); 50 µg/mL in acetone:methanol solution (2:3)) was added per well to generate control and +POP experiments. Plates were placed in a humidified incubator maintained at 37ºC and 5% CO_2 (Thermo Scientific, Midi 40 CO_2 Incubator, model: 3404) for 24 h. All media was drawn off after 24 h and frozen at -20 ºC. Explants were snap frozen in liquid nitrogen and stored at -80 ºC.

Metabolite measurement

Glucose, lactate and glycerol concentrations were measured in media from explant experiments using Randox (County Antrim, UK) kits (glucose: GL364, lactate:

197 LC2389, glycerol: GY105) and standards in an RX Monza (Randox) Clinical Chemistry
198 analyser (Model: 328-14-0914) as described previously[91]. Internal quality control
199 measurements lay within < +/− 15%. Intra and inter assay variability for sample
200 analysis was < 5%. Mean variation between replicates from the same individual and of
201 the same nutritional state and tissue type for the three metabolites analysed were as
202 follows; glucose 26.6% (range 0.6-144%), lactate 46.9% (range 3-164%) and glycerol
203 19.8% (range 0.5-106%). Rates of glucose removal and accumulation of lactate (indices
204 of glycolysis) and glycerol (indices of lipolysis) were calculated per 100 mg tissue^{-1}
205 hour^{-1}.
206
207 *POP analysis*
208 Extraction and detection of POPs took place at the Center for Analytical
209 Research and Technology (CART),University of Liège, Belgium, using standard
210 methods (supporting information SI 1). The 6 mm biopsies yielded 385 ± 7 (s.e.) mg
211 blubber tissue per sample. Six non Dioxin-Like PCBs (NDL-PCBs) (28, 58, 101, 138,
212 153, 180), eight Dioxin-Like PCBs (DL-PCBs) (105, 114, 118, 123, 156, 157, 167, 189)
213 and nine PBDEs (28, 47, 66, 85, 99, 100, 153, 154, 183) were analysed at CART in the
214 Department of Chemistry. Four OCPs (o,p'-DDT, p,p'-DDT,
215 dichlorodiphenyldichloroethane (DDD), dichlorodiphenyldichloroethylene (DDE)) were
216 analysed at CART in the Laboratory of Animal Ecology and Ecotoxicology (LEAE).
217 POP concentrations were measured in ng g^{-1} lipid, summed into ∑NDL-PCBs,
218 ∑DL-PCBs, ∑PBDEs and ∑DDXs (o,p'-DDT, p,p'-DDT, DDD, DDE) and log
219 transformed prior to statistical analysis.
220
221

Statistical analysis

Analyses were performed using the statistical package R 3.4.1[99]. T-tests were used to compare log transformed POP concentrations between feeding and fasting samples. Pearson's correlation coefficient was calculated between all four ∑POP types (log transformed ∑NDL-PCBs, ∑DL-PCBs, ∑PBDEs and ∑DDXs). As all POP classes were highly significantly correlated, we explored the effects of each POP class separately. Generalised additive mixed models (GAMM)[100] were used to identify the variables that best explained glucose uptake, lactate production and glycerol production. Explanatory variables explored in these models included log transformed ∑NDL-PCBs, ∑DL-PCBs, ∑PBDEs and ∑DDXs in the blubber at time of sampling (each POP class tested separately in each model), the mass of the pup, the tissue depth (inner or outer), the nutritional state at sampling (feeding or fasting), the experimental conditions (control or +POP) and the pup's sex. Interaction terms between nutritional state, tissue type, experimental conditions and POP concentrations were also investigated (see SI Table 1). The model was fitted using the multiple generalized cross validation library *mgcv*[101]. The identities of individual pups were fitted as random effect smooths[102] to account for the same individual generating multiple explants and any variation between replicates. The smoothing parameters were set by maximum likelihood to reduce the risk of overfitting[103]. The models were fitted with a Gaussian error distribution. Glucose data were log transformed and glycerol data were square root transformed.No transformation was needed for lactate data. Model selection was performed by backwards stepwise elimination through examination of R^2 values, Akaike's information criterion (AIC) values, dAIC, Akaike's weights, QQ and residual plots

Results and Discussion

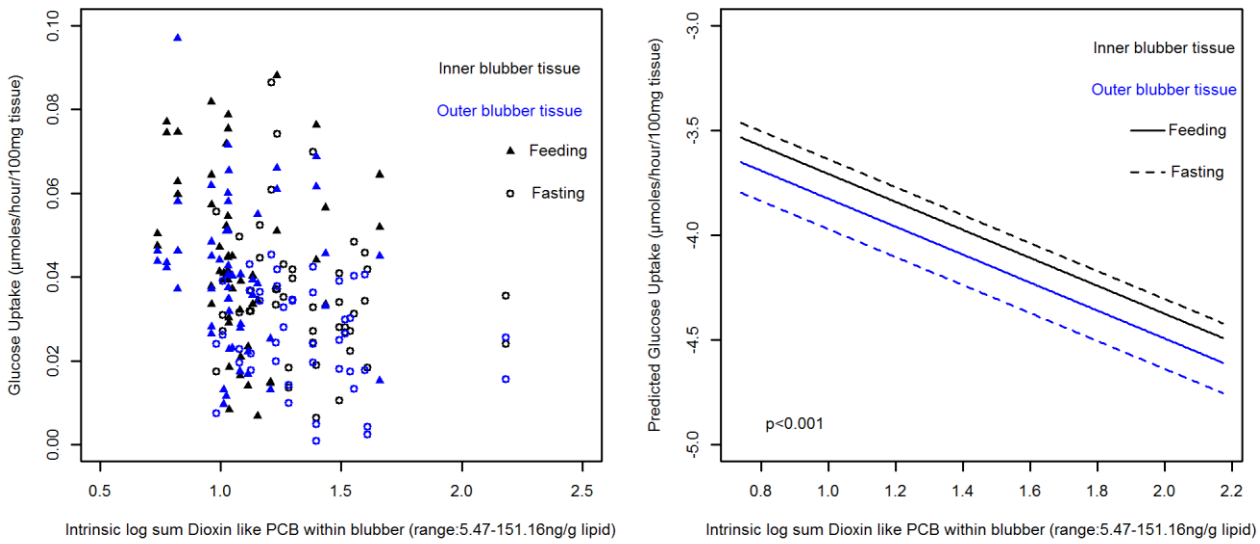
POP concentrations

Mean concentrations and ranges of the summed POPs in grey seal pup blubber are given in Table 1.

Table 1. Means and ranges for study pup masses and fat content of 6mm biopsies, with median concentrations and ranges of POPs in grey seal pup blubber in feeding and fasting states.

	Feeding mean	Feeding range	Fasting mean	Fasting range
Pup mass (kg)	41.9	31.4 – 52.6	40	32.8 – 50.4
Biopsy fat content (% lipid)	78.5	67.8 – 85.5	82.9	56.3 – 88.9
	Feeding median	Feeding range	Fasting median	Fasting range
∑NDL-PCB (ng g^{-1} lipid)	354.29	194.2 – 790.5	541.73	302.5 – 1253.8
∑DL-PCBs (ng g^{-1} lipid)	10.78	5.5 – 45.6	19.78	9.6 – 151.2
∑PBDE (ng g^{-1} lipid)	13.81	6.4 – 57.9	20.2	10.2 – 70.1
∑DDX (ng g^{-1} lipid)	169.09	98.5 – 420.8	209.56	128.4 – 345.7

257 Higher POP levels when pups were fasting (Welch's two sample t-test, $p < 0.001$
258 for all log transformed POP classes) are consistent with previous work showing that
259 lipid mobilisation during fasting concentrates more lipophilic POPs in remaining fat
260 tissue[50,51,53]. Blubber may thus be more vulnerable to disruptive effects of contaminants
261 during fasting if the POPs can interact with cellular machinery to exert negative effects.
262
263 *Glucose uptake*
264 ∑DL-PCBs, experimental conditions, tissue depth, and nutritional state best
265 explained variation in glucose uptake, along with an interaction between tissue depth
266 and nutritional state (GAMM: $R^2 = 0.36$, SI Table 2). ∑NDL-PCBs, ∑PBDEs and
267 ∑DDX had no significant impact on glucose uptake (SI Table 1). Uptake was higher in
268 inner compared to outer tissue ($p < 0.001$). Glucose uptake was lower during fasting
269 than feeding in outer blubber ($p = 0.035$), but did not differ between nutritional states in
270 inner tissue (Figure 1). In both inner and outer tissue, glucose uptake was inversely
271 related to ∑DL-PCBs ($p < 0.001$) (Figure 1) and the slope of the relationship did not
272 depend on nutritional state. Acute exogenous POP exposure increased glucose uptake
273 compared to controls, (GAMM: $R^2 = 0.36$, $p < 0.001$, SI Table 2, Figure 2), and the
274 increase was not affect by nutritional state or tissue depth. Doses of each PCB congener
275 given in the explant POP exposure conditions, with the estimated percentage change for
276 each PCB type measured in blubber are given in SI Table 3. A boxplot of glucose
277 uptake data during different nutritional states and tissue depths is provided in SI Figure
278 1.
279



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281 Figure 1. Scatter plot (left panel) and prediction line plot (right panel) showing the

282 impact of intrinsic ΣDL-PCBs on glucose uptake in blubber from grey seal pups,

283 comparing inner (black) /outer (blue) tissue and feeding (filled triangles or solid lines)

284 /fasting (open circles or dashed lines) states (n = 27 feeding and n = 23 fasting pups).

285 Individual was included as a random effect in the model.

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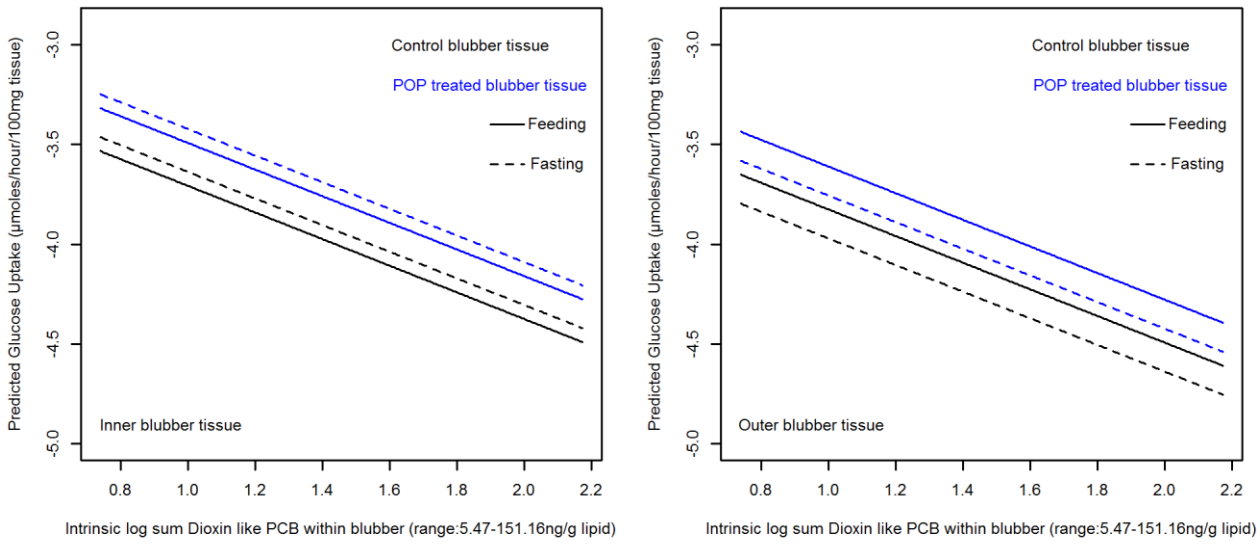

287

288 Figure 2. Prediction line plots showing the impact of exposure to acute exogenous POPs

289 on glucose uptake in blubber from grey seal pups, comparing inner (left panel) and

290 outer (right panel) tissue and feeding/fasting states across the range of intrinsic ΣDL-
291 PCBs in the blubber. Individual was included as a random effect in the model.
292
293 These data show for the first time that metabolic activity in phocid blubber is
294 altered by the POPs accumulated *in utero* and during suckling. POPs have been
295 postulated to be 'obesogens' from epidemiological data in humans[24]. Experimental
296 studies have demonstrated that POPs can alter adipocyte function in rodents and
297 human cell lines[25,104]. POPs are associated with altered adipose and liver expression of
298 metabolic pathway genes in adult polar bears[55,66] and ringed seals (*Pusa hispida*)[63,64],
299 but the functional consequences of such changes for fat tissue have not been identified.
300 Recent experimental studies that replicate environmental or physiological POP
301 concentrations and congener mixtures provide causal evidence of their
302 immunological[105] and adipogenic effects[91]. Our work builds on these findings by
303 showing that existing POP concentrations in blubber tissue, generated *in utero* and
304 through lactational transfer, can alter metabolic properties of adipose tissue in young,
305 developing seals.
306 Our data are consistent with experimental studies showing PCB 77, 153 and
307 dioxin reduce glucose and insulin tolerance in mice, guinea pigs (*Cavia porcellus*),
308 rabbits (*Oryctolagus cuniculus*) and in human cell lines[69-77]. PCBs are also associated
309 with dysfunctional glucose homeostasis in humans[78-81]. Here, only ΣDL-PCBs altered
310 glucose uptake in blubber explants, consistent with an aryl hydrocarbon receptor (AhR)-
311 dependent pathway in seals, as seen in other species[71,78]. POP-induced reduced glucose
312 use is accompanied by a fall in glucose transporter, GLUT4, mRNA expression and
313 GLUT1 protein levels in other animals[75], and may also occur in seals. The opposite
314 effects of an exogenous POP exposure to the intrinsic ΣDL-PCB levels on glucose

315 uptake may reflect the difference between short (overnight) *in vitro* and long-term (*in*
316 *utero* and over 15 days of suckling) exposure, which may produce opposing effects on
317 membrane fluidity and transport properties[106]. Alternatively, either the DL-PCB dose in
318 the acute exposure experiments may have been too low to affect glucose uptake (SI
319 Table 3) or the exogenous POP cocktail produces different effects from the *in vivo*
320 contaminant mixture. We cannot distinguish between these possibilities from our data.
321 Lower glucose uptake caused by DL-PCBs is likely to reduce fat deposition rate,
322 which may be problematic for pups laying down fat while suckling. However, fat
323 deposition may increase if acute exogenous POP exposure increases glucose uptake.
324 Indeed, short-term exposure to a POP cocktail from polar bear blubber induced
325 adipogenesis in a mouse pre-adipocyte cell line[66]. The timecourse of POPs on both
326 adipogenesis and lipogenesis needs to be explored.
327 NDL-PCBs, DDX and PBDEs in blubber did not affect glucose uptake, despite
328 evidence of their impacts on glucose homeostasis in other animals [82-87, 107]. Our data
329 suggest that the negative relationship between PBDEs and first year survival of grey
330 seal pups[104] is not driven by direct alterations to adipose metabolic properties. PBDEs
331 may alter adipose function indirectly through endocrine disrupting effects, for example,
332 by altering thyroid or insulin function[86, 87, 108, 109], highlighting the need for improved
333 understanding of energy balance regulation in seals, and its disruption by POPs.
334 Glucose uptake was higher in inner tissue under all conditions, suggesting
335 deeper blubber has greater metabolic activity than superficial tissue. This finding is
336 consistent with regional stratification in structure; the theorised role of superficial
337 blubber in thermoregulation; the possibility of smaller, more numerous adipocytes that
338 are more metabolically active[92] in deeper tissue; and the observation that deeper blubber

339 contains higher glycolytic enzyme levels compared to superficial blubber in fin whales
340 (*Balaenoptera physalis*[92]).

341 Glucose uptake fell as the animals transitioned from feeding to fasting in outer
342 blubber, but not in inner blubber. This likely reflects high levels of fat deposition
343 throughout the blubber during feeding, followed by cessation of fat accumulation during
344 fasting. Metabolic rate falls in fasting pups [42,110], and this is likely reflected in reduced
345 demands by all tissues, including blubber. Lower sensitivity to residual insulin present
346 in stripped FBS may explain lower glucose uptake and greater decrease in outer tissue
347 from fasting animals, a state in which many animals become less sensitive to the effect
348 on insulin of glucose uptake [111-113]. The lack of decreased glucose uptake in inner
349 blubber in fasting animals is surprising. It is however plausible that cell membrane
350 expression of GLUT4 and/or insulin -independent GLUT1, are reduced to a greater
351 extent in outer compared to inner blubber during fasting. Indeed, fasting northern
352 elephant seals (*Mirounga angustirostris*) maintain expression of GLUT4 in adipose cell
353 membranes[114].

354 Previous studies of metabolic effects of POPs on marine mammals have
355 focussed on adults[55,63,64,66]. Concentrations of POPs in adult seals are an order of
356 magnitude greater than those found in pups, with adult seal blubber containing several
357 thousand ∑PCBs (ng/g)[115] compared to the levels detected in the pups here, which were
358 in the hundreds of ng/g range (Table 1). Here, we have shown that pups have already
359 accumulated high enough POP levels in their blubber to impact on adipose function at
360 less than three weeks of age, when the ability to generate and regulate blubber is a key
361 factor for survival[116]. Growing and dividing adipocytes are more vulnerable to
362 disruption by DL-PCBs because they have higher AhR levels than mature,
363 differentiated adipocytes[117,118]. The sensitivity to POP-induced alterations to adipose

364 tissue metabolism is therefore likely to be greatest in young marine mammals, which
365 are exposed to large quantities of POPs from their mothers[47,50] and are likely to have a
366 high proportion of proliferating pre-adipocytes as a result of rapid fat deposition[119]. The
367 energy balance and survival consequences of reduced glucose uptake in young pups
368 now needs to be explored.
369
370 *Lactate production*
371 ∑DL-PCBs, tissue depth, nutritional state, pup sex and interactions between
372 ∑DL-PCBs and nutritional state, and tissue depth and nutritional state were retained in
373 the model that best explained lactate production (SI Table 1 and 2). ∑NDL-PCBs,
374 ∑PBDEs and ∑DDX had no significant impact on lactate production (SI Table 1).
375 Lactate production increased with DL-PCBs in feeding pups, but decreased with DL-
376 PCBs when pups were fasting (GAMM: $R^2 = 0.29$, $p = 0.003$; SI Table 2, Figure 3).
377 Lactate generation was higher in males than in females ($p = 0.05$) and higher in inner
378 tissue than outer tissue for any given DL-PCB load in any given nutritional state ($p =$
379 0.001) (Figure 3). The difference between inner and outer tissue tended to be greater
380 during feeding than during fasting ($p = 0.055$). Lactate generation was not impacted by
381 acute exogenous experimental POP exposure. A boxplot of lactate generation/uptake
382 data during different nutritional states and tissue depths is provided in SI Figure 2.
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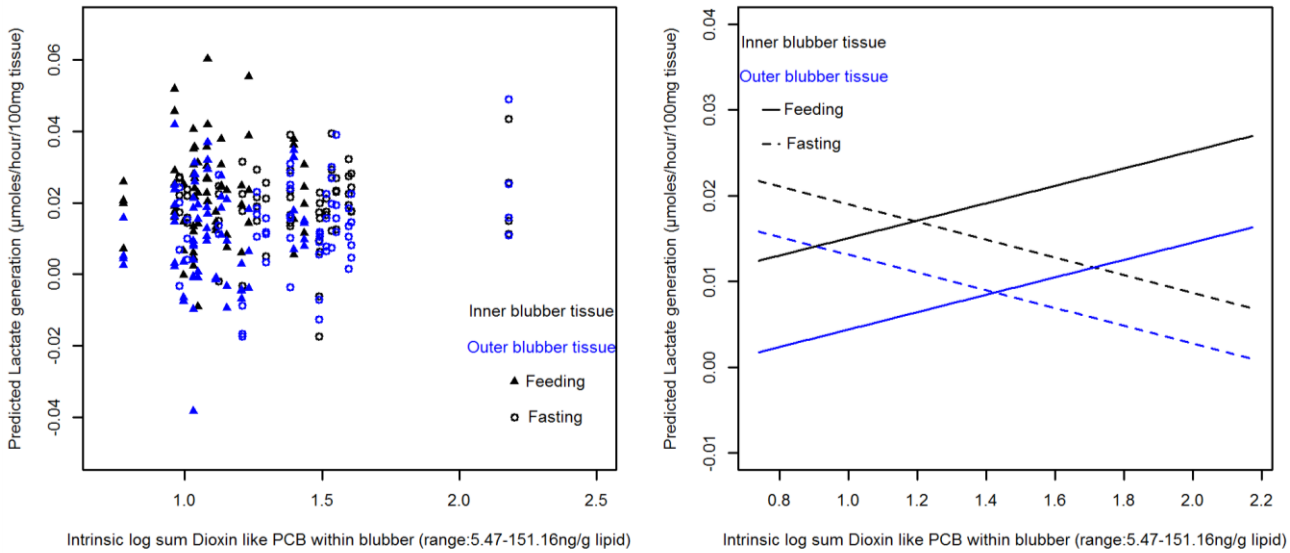

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385 Figure 3. Scatter plot (left panel) and prediction line plot (right panel) showing the
386 impact of intrinsic ΣDL-PCBs on lactate generation/uptake in blubber from male grey
387 seal pups (n=34), comparing inner (black) /outer (blue) tissue and feeding (filled
388 triangles or solid lines) /fasting (open circles or dashed lines) states. Individual was
389 included as a random effect in the model.

390

391 Lactate production is a major route of glucose disposal in fat tissue[67]. However,
392 the inhibitory effect of DL-PCBs on glucose uptake is only consistent with lactate
393 production during fasting. During feeding, lactate production increased with increased
394 DL-PCBs in the face of reduced glucose uptake. One explanation is that the electron
395 transfer chain experiences increased push force during suckling, in combination with
396 inhibition by DL-PCBs[120,121], which could elevate lactate production through increased
397 reliance on glycolysis. Suckling pups with higher DL-PCB burdens could thus
398 experience greater risk of metabolic acidosis from higher adipose lactate production,
399 with attendant risk of adipose dysfunction, greater demand on liver gluconeogenesis by

400 to process the lactate, and thus heightened energetic costs that could impact on
401 efficiency of mass transfer and fat accretion during suckling.
402 Adipose lactate production is a direct consequence of lactate dehydrogenase
403 (LDH) activity[122]. LDH activity can fall during fasting[123], and LDH gene expression
404 and enzyme activity also differ substantially between adipose depots in rats[122]. LDH
405 activity can also be modulated by PCB exposure in rat and dog liver, and the effect is
406 dose dependent[124,125]. Shifts in LDH expression and activity may thus contribute to the
407 differences in lactate production between depths and nutritional states seen here in
408 response to DL-PCBs. Higher lactate generation in inner tissue and the greater
409 difference between regions during feeding may be a result of greater glucose uptake and
410 metabolic activity in inner blubber during suckling. Regional differences in metabolic
411 properties may also relate to adipocyte size[126].
412 Higher lactate production by adipose from males than females has not been
413 reported, either in adult seals[91] or in humans and rodents[122] and may result from the
414 small number of females here. However, since adult females accumulate fat faster than
415 adult males[127] and female juvenile northern elephant seals spare fat more effectively
416 than males[128], it is possible that some metabolic differences are present in pups' blubber
417 prior to overt size dimorphism. For example, females may use more lactate to synthesise
418 FA[129] than males, releasing less net lactate.
419
420 *Glycerol production*
421 Nutritional state, an interaction between tissue depth and nutritional state, and an
422 interaction between nutritional state and intrinsic blubber DDX were significant terms
423 in the best model to explain glycerol production (SI Table 1 and 2, Figure 4). In
424 addition, extrinsic POP treatment, mass and sex were retained as non significant

variables. Unexpectedly, glycerol production, was higher during feeding than fasting (GAMM: $R^2 = 0.74$, $p < 0.001$ Figure 4). In addition, lipolysis was higher in outer than in inner tissue during feeding, whereas inner tissue had a higher lipolytic rate than outer tissue in the fasted state ($p = 0.0003$; SI Table 2). Finally, lipolytic rate increased with intrinsic blubber DDX in fasting animals, whereas the opposite relationship between DDX and lipolytic rate occurred in feeding pups ($p < 0.0001$, Figure 4). A boxplot of glycerol generation data from different nutritional states and tissue depths is provided in SI Figure 3.

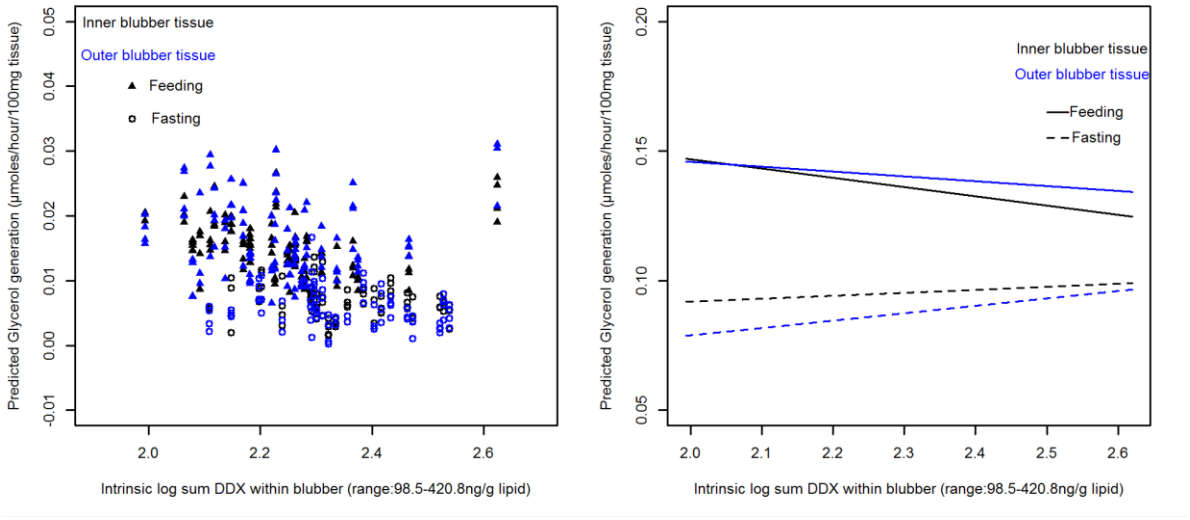

Figure 4. Scatter plot (left panel) and prediction line plot (right panel) showing the impact of intrinsic ΣDDX on glycerol generation in blubber from grey seal pups, comparing inner (black) /outer (blue) tissue and feeding (filled triangles or solid lines) /fasting (open circles or dashed lines) states (n = 27 feeding and n = 23 fasting pups). Individual was included as a random effect in the model.

POPs, such as dioxin and related compounds, and OCPs typically produce 'wasting syndrome' at high doses[32-35], likely due to increased WAT lipolysis[130]. In contrast, some studies on rat adipose or rodent cell lines show no effect of DDT or DDE

444 on lipolysis at lower doses[131,132]. Our data show that lipolysis is increased in blubber of
445 fasting grey seal pups in response to intrinsic tissue levels of DDT and its metabolites,
446 but decreased in feeding pups. These data suggest, that, as in bats, seal pups in negative
447 energy balance may be most vulnerable to the presence of DDX in their tissues[34].
448 Interestingly, the increase in lipolysis is greater in outer tissue, which is proposed to be
449 important for insulation [56-61], such that DDT may negatively affect both
450 thermoregulatory and metabolic capability of blubber during fasting. A reduction in
451 lipolytic rate with DDX in feeding animals could reflect inhibition of tissue turnover of
452 FFA, increased lipogenesis or a shift towards lactate production rather than glycerol
453 generation during glycolysis[68].
454 Lower glycerol production during fasting compared to feeding contrasts starkly
455 with the typical fasting increase in lipolysis[133,134]. Regional and nutritional differences
456 may result from depth changes in metabolic activity and adipocyte size[135]. Lower
457 glycerol production in fasting pups could also arise from a reduction in aldolase
458 activity[136]. In addition, higher lipolytic rates in feeding animals may reflect their higher
459 metabolic rate [42,110], faster rates of triglyceride turnover and/ or greater production of 3
460 carbon (3C) intermediates to protect tissues against high fuel availability and provide
461 fuel for actively growing tissue elsewhere[68,122]. High rates of glycerol production in
462 feeding animals could protect adipocytes from metabolic acidosis, minimise
463 overproduction of oxidising equivalents and maximise energy efficiency[68,137].
464
465 *Value and wider application of the explant method*.
466 Wildlife species are facing increasing anthropogenic threats that can limit their
467 ability to find food and store fat as an energy reserve for lean times. Constrained,
468 fragmented or reduced foraging ranges[138] and decreased prey availability[139,140] limit

opportunities for fat accumulation, and force individuals to rely on adipose stores more frequently to sustain them until the next feeding opportunity. Understanding the impact of natural and anthropogenic stressors on energy balance requires experimental interventions that are practically and ethically challenging to accomplish in marine mammals and other wildlife species. *In vitro* methods are increasingly being explored as alternatives to whole animal work and lethal sampling. Prior efforts have generated viable skin tissue samples for culture via dermal punches and biopsy darts in both marine[141,142] and terrestrial[143] wildlife species. Given the importance of fat tissue to the health and survival of wildlife in rapidly changing environments, there is a critical need for methods to better understand their adipose physiology. We previously used blubber explants from wild seals,which were returned to tissue culture facilities in a mainland laboratory[91]. Here, we collected and maintained viable blubber explants for 24 h in a remote field laboratory, with only core tissue culture equipment. The methodology outlined here could therefore be adapted for use in other wildlife species in remote field locations or where alternative *in vitro* methods are not available.

Outlook

Our explant method clearly shows promise in better understanding adipose tissue responses to contaminants and can be expanded beyond the parameters measured here. Although establishment of cell lines would permit a wider range of exposures, including time courses, dose responses and mixtures over longer duration experiments, such approaches have been hampered by the absence of typical proliferation and differentiation responses in primary cells from non-model species[53]. A focus on understanding the requirements of such cell types and generation of cell lines would greatly facilitate progress in toxicological studies on adipose from wildlife species.

In summary, we have shown that dioxin-like PCBs disrupt adipose glucose uptake and lactate production, and DDX disrupt lipolysis in grey seal pups, using an experimental explant approach. Our results are particularly important because they demonstrate POP-induced alterations to adipose tissue function in young animals in the first weeks of life, for which rapid fat accumulation during suckling and mobilisation during fasting are vital for survival. They also highlight that different POPs may impact different metabolic characteristics, and that their effects are modified by nutritional state and tissue depth, producing complex effects on tissue function. Wider screening of metabolic properties of cells and tissue from non-model organisms is key to identify energy-balance disrupting effects of legacy and emerging contaminants. Our methods and findings also have wider application in understanding drivers of juvenile survival of other top marine predators, particularly in species that simultaneously experience high POP loads and rapid alterations in prey availability and habitat.

Acknowledgments:

We would like to thank Simon Moss, Holly Armstrong and everyone who assisted with colony observations and sample collection on the Isle of May during the 2016 season. We would like to thank Scottish National Heritage for the permit to work on the Isle of May and the skipper and crew of the Osprey and May Princess for help with gear and personnel transport. We would also like to thank Dr Georges Scholl, Catherine Adam and all of the staff at the University of Liège who provided training and assistance while KJR was in the lab processing samples for POP detection, and Dr James Turton at Abertay University for advice on adipocyte tissue cell culture techniques.

The long term program of research on grey seals at the Isle of May was funded by the SMRU national capability grant (SMRU 1001). KAB was funded by NERC grant NE/M013723/1 and AJH and KJR were funded by NE/M01357X/1 for this project. The funding bodies had no role in the design of the study, the collection of samples, the analysis of samples or data and the interpretation of the data. The authors declare that they have no competing financial interests.

Supporting Information

SI 1. Methodological details for POP detection.

SI Table 1. Candidate GAMM models exploring different summed POP types, potential interactions and the top models in the selection process.

SI Table 2. Model output from all final GAMMs analysing metabolic characteristics in explant experiments with their standard errors, estimates and p values. If predictor variables have parentheses, this indicates the tested group.

SI Table 3. Doses of each PCB type given in the explant PCB culture conditions, with the estimated percentage change for each PCB type measured in blubber tissue. ∑DL-PCB within total dose: 1.6ng. ∑NDL-PCB within total dose: 202.6ng.

SI Figure 1. Glucose uptake in inner and outer blubber tissue from feeding and fasting grey seal pups (n = 27 feeding and n = 23 fasting pups) with median, upper and lower quartiles, 1.5 x interquartile range and outliers shown. Significant differences between

groups are detailed in the main text, as not all significant parameters and interactions influencing glucose uptake are plotted. Individual was included as a random effect in the model.

SI Figure 2. Lactate production in inner and outer blubber tissue from feeding and fasting grey seal pups (n = 27 feeding and n = 23 fasting pups) with median, upper and lower quartiles, 1.5x interquartile range and outliers shown. Significant differences between groups are detailed in the main text, as not all significant parameters and interactions influencing lactate generation/uptake are plotted. Individual was included as a random effect in the model.

SI Figure 3. Glycerol generation in inner and outer blubber tissue from feeding and fasting grey seal pups (n = 27 feeding and n = 23 fasting pups) with median, upper and lower quartiles, 1.5x interquartile range and outliers shown. Significant differences between groups are detailed in the main text, as not all significant parameters and interactions influencing glycerol generation are plotted. Individual was included as a random effect in the model.

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